INSIDE THE CELL

A New Paradigm for Unit Operations and Unit Processes?

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Traditionally the chemical engineering paradigm for designing industrial plants has been to separately consider unit operations and unit processes. Although these terms are not prevalent in current curricula, courses in separations, reaction engineering, polymer engineering, etc., reflect this traditional view.

The usual scheme of process development starts at the laboratory bench. And with experimentation and exploration, conditions are optimized and then the process goes through a scale-up procedure to reach commercial production needs. Recent advances in MEMS technologies, however, have led to the implementation of Lab-On-a-Chip devices such as the integrated DNA analysis chip developed by Burns, *et al.*[1] (see Figure 1). These analytical devices bring together various elements of unit processes such as separation steps, reaction steps, and process control (detection and sensing procedures). The capability for miniaturization and integration has led some chemical engineers (*e.g.*, Klavs Jensen) to envision an entirely new paradigm for production methods, *i.e.*, scaling down instead of scaling up. Figure 2 from Professor Jensen's Web site succinctly illustrates this contrast in paradigms.

Given the immense efforts in MEMS and nanotechnology toward miniaturization and integration, one can readily speculate about potential future methods for carrying out chemical processes through the application of these technologies. The organizational structure of biological cells could have important lessons and impact in this regard. As the extensive structural and operational characteristics of the biological cell are being revealed, it is becoming clear that biological systems have evolved with a much more integrated design paradigm of processes and operations than the traditional chemical engineering approach.

Jerome Schultz *received his B.S. and M.S in chemical engineering from Columbia University, and his Ph.D. in biochemistry from the University of Wisconsin in 1958. He started his career in the pharmaceutical industry (Lederle Laboratories) then joined the University of Michigan, where he was chairman of the Department of Chemical Engineering. He spent two years at the National Science Foundation as deputy director of the Engineering Centers Program. In 1987 he joined the University of Pittsburgh as director of the Center for Biotechnology and Bioengineering, and was the founding chairman of the Department of Bioengineering—a nationally ranked degree program in bioengineering. He recently spent a year at NASA's Ames Research Center as a senior scientist in their Fundamental Biology Program. In 2004 Dr. Schultz joined the faculty at the UC Riverside as the director of the newly formed Bioengineering Program and the Center for Bioengineering.*
He is a member of the National Academy of Engineering, a Fellow of the American Association for the Advancement of Sciences, Editor of Biotechnology Progress, and was a founding Fellow and President of the American Institute for Medical and Biological Engineering.

New advances in genomics, proteomics, metabolomics, cell signaling, and control have allowed the documentation of the thousands of species and interactions that comprise the internal milieu of cells. This vast amount of information has allowed the harnessing of biological cells for many purposes such as preparation of many biologics (*e.g.*, insulin and EPO [erythropoietin, monoclonal antibodies]), as well as the use of cells for detoxification of herbicides.

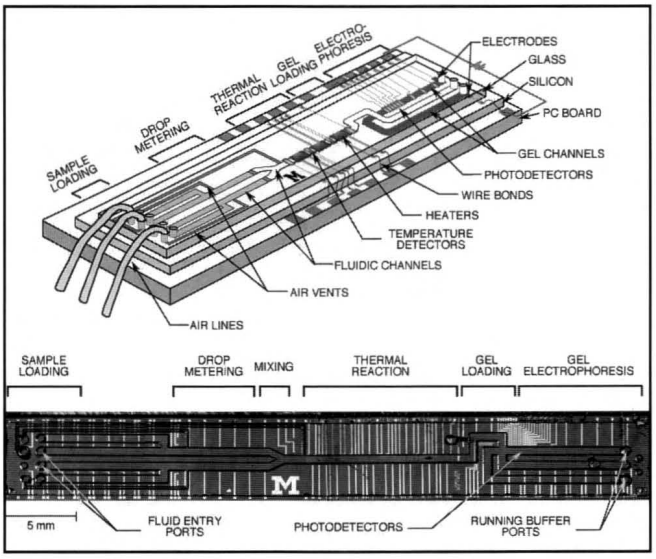


Figure 1. *An example of a Lab-On-a-Chip device. This is a DNA analysis system devised by Mark Burns and associates.[1] Various "unit operations" including metering, mixing, reactions, separations, and detection are combined in a single device.*

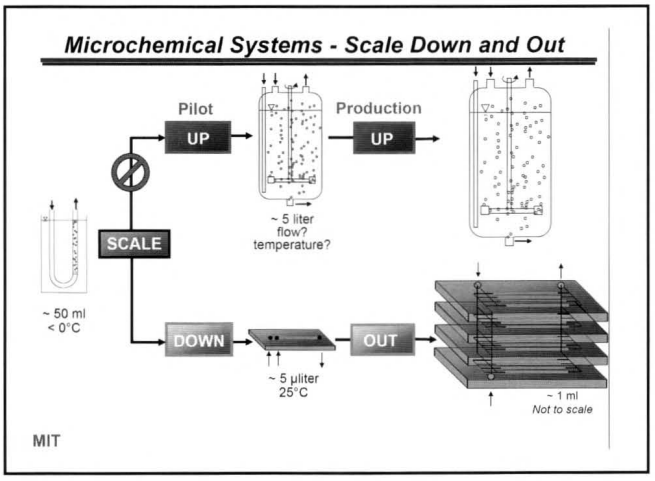


Figure 2. *Klavs Jensen's concept for a new paradigm in chemical process development that utilizes the multiplexing capabilities of MEMS technology to carry out integrated synthesis and separation operations in the same unit.*

Much of our thinking related to the future of biotechnology is based on our appreciation of biological systems deduced from the dissection and separation of the components of cells such as enzymes, signaling proteins, antibodies, RNA, and DNA. Much of the richness of biological systems, however, resides in the structural features of cells. To date, many of the structural elements that have been deduced from electron micrographs are categorized as organelles. Some of the classes of organelles that have been identified include the nucleus, mitochondria, lysosomes, peroxisomes, vesicles, chloroplasts, and golgi (Figure 3). It is clear that cells are not a bag of enzymes and substrates, *i.e.*, a CSTR.

Although the morphology of these structural elements is fairly well characterized by electron microscopic methods, the functional and dynamic biological/chemical processes that are taking place in these structures are not well understood at all. Early hints from the study of some of these organelles have revealed that biology does not separate unit processes from unit operations, but rather integrates them. For example, in chloroplasts the capture of photons and fixation of carbon dioxide into carbohydrates simultaneously results in photolysis—the separation of protons and oxygen evolution. Ribosomes integrate the genetic code and protein synthesis.

Most organelles are known to be complex multi-membranous structures, but the composition and detailed organization of these units are not known. One reason for the lack of detailed understanding is that the typical dimensions of these structures is on the order of nanometers and thus below the resolution of optical microscopes. So they cannot be visualized in detail while in a normal functional mode. This lack of

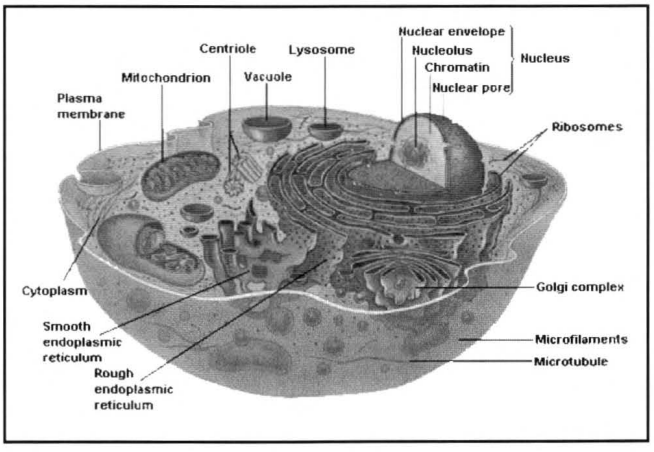


Figure 3. *Diagramatic illustration of the various structures within a cell illustrating the complex structures inside of cells that are responsible for much of the biosynthetic activities of living systems.*

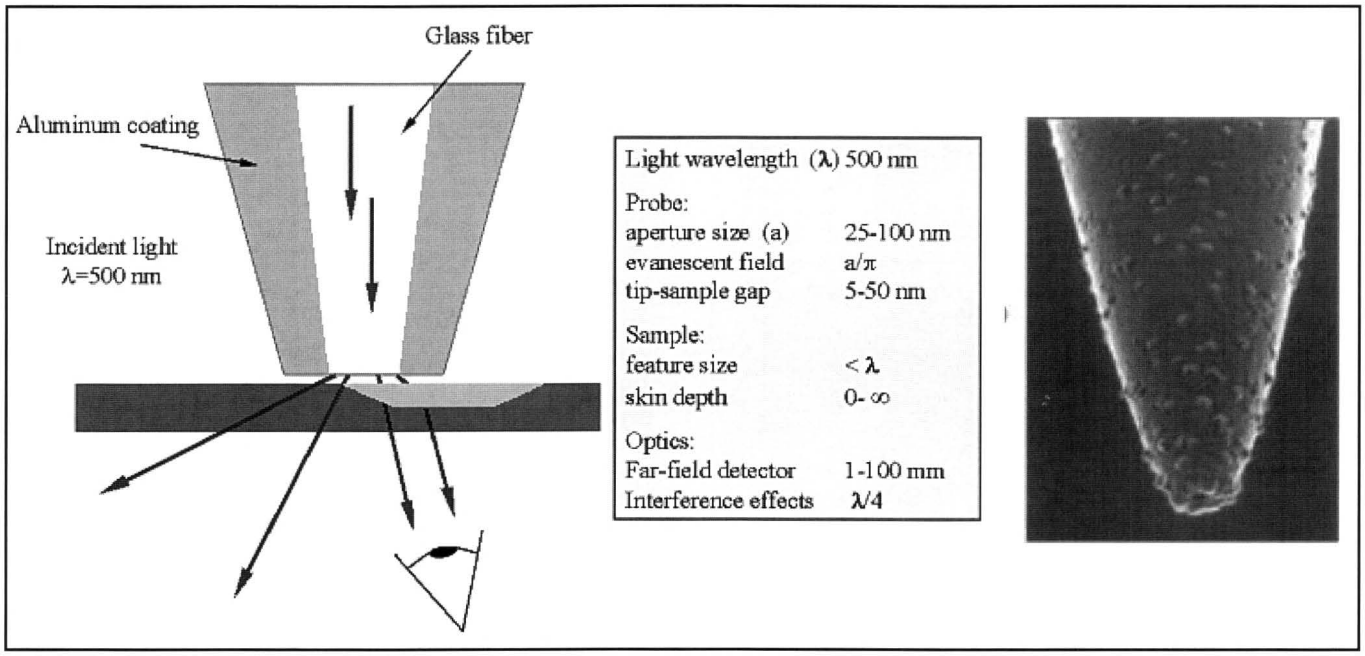


Figure 4. *Operational characteristics of near field microscopy (left). Close-up of the optical scanning probe (right). This is one of the devices that will allow the visualization of structures within living cells and their behavior.*

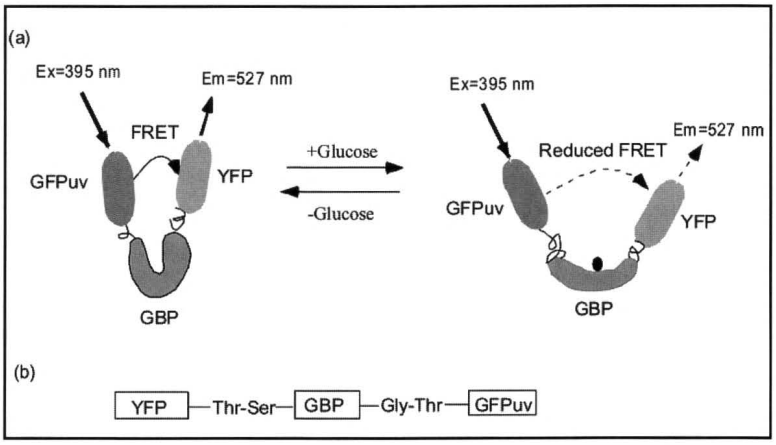


Figure 5. *Glucose-responsive protein engineered from a glucose binding protein from E. coli, and two different green fluorescent proteins. This type of structure can be introduced into a cell via a plasmid to result in the biosynthesis of the sensor protein within the cell.* ***(a)*** *In the absence of glucose the two fluorophores are in close proximity and exhibit fluorescent energy transfer (FRET) (left). In the presence of glucose the protein opens, and FRET is reduced.* ***(b)*** *Structure of the fusion protein.*[10]

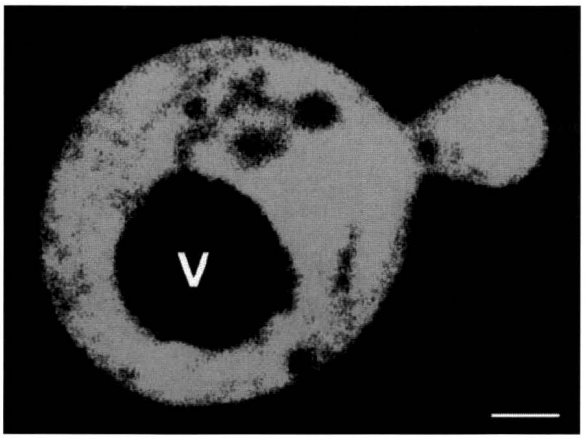
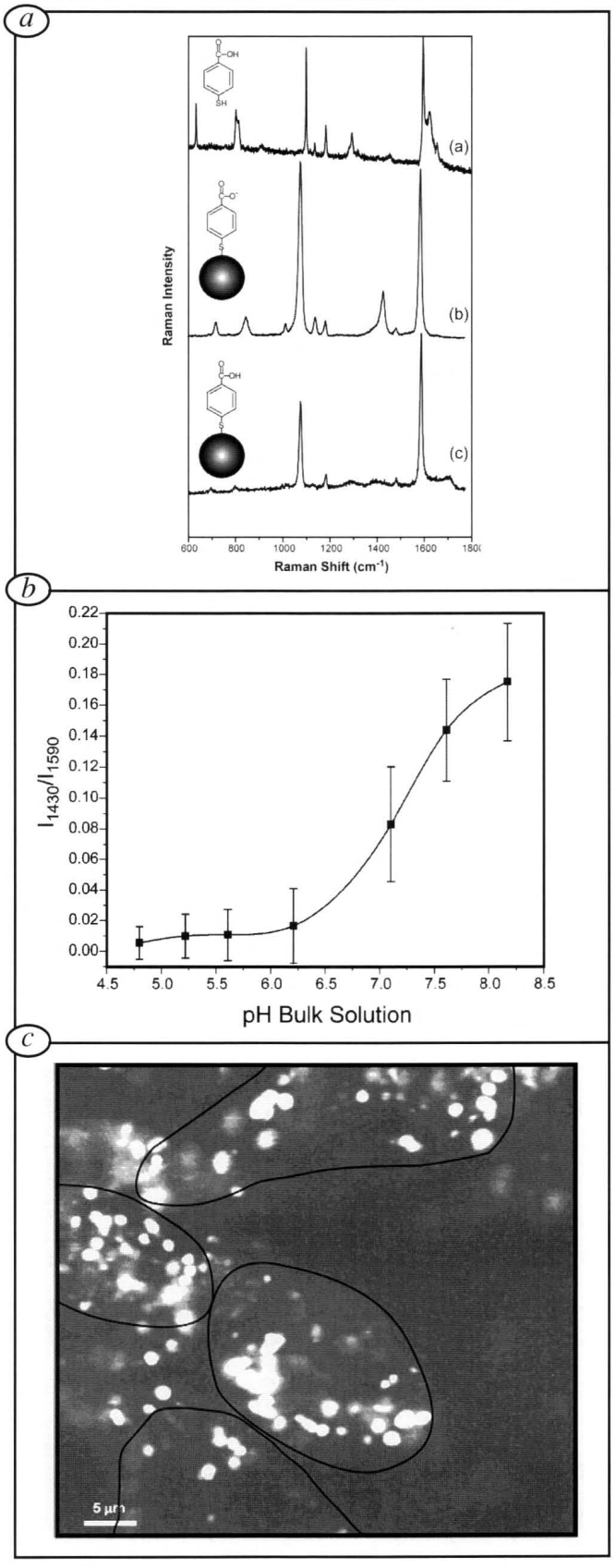


Figure 6. *Confocal image of a yeast cell containing a maltose responsive protein similar to that illustrated in Figure 5. The intensity of fluorescence is indicative of the concentration of maltose in various regions of the cell. By incorporating various indicator proteins of this type within cells, one could monitor the dynamics of biosynthesis of specific biomolecules in space and time. V is a vacule. (Bar = 1 micron).* [11]

knowledge has hampered our ability to mimic these biological systems for chemical processes.

Hope is on the way, however. To deal with this issue, indirect methods are under active development to elucidate mechanisms of the functioning of organelles.

Many new instrumental techniques are being developed to provide some real-time measurements of the behavior of subcellular structures. These techniques include confocal microscopy,[2] two-photon microscopy,[3] and optical coherence tomography.[4] Near field microscopy,[5] Figure 4, allows the



visualization of structural elements near the surface of the cell. Basically an optical fiber is drawn down to diameters less than the wavelength of light and placed in contact with the cell's outer membrane. The probe is scanned across the cell membrane to provide a map of structures just beneath the membrane surface.

Several clever concepts based on various reporter techniques have also been described recently that are beginning to give specific dynamic data on intracellular events. The rapidly expanding knowledge base on the structure and properties of green fluorescent proteins has opened up many opportunities for the protein engineering of intracellular probes. A multitude of techniques is available for incorporating plasmids for these proteins into cells. These reporter indicators can be either freely mobile within the cell or localized in specific structures.[6, 7]

Roger Tsien and his group[8, 9] have pioneered the use of green fluorescent proteins as functional probes for biomolecules within cells based on the technique of fluorescence energy transfer (FRET). One recent application of this approach has been to monitor sugar concentrations within cells. We[10] engineered a fusion protein consisting of a glucose-binding protein and two different green fluorescent proteins as shown in Figure 5. The sugar-binding moiety undergoes a conformational change when glucose binds, such that it changes the distance between the GFP and YFP in a manner that results in a change in FRET. Fehr, *et al.*,[11] incorporated a similar maltose-binding protein into yeast cells. Using confocal microscopy, they were able to monitor the distribution of maltose throughout the cell, Figure 6.

Other techniques for monitoring the concentration of materials within cells are based on inserting tiny "biosensor" particles within cells. Raoul Kopelman and colleagues[12] have designed various materials called "PEBBLES" for measuring oxygen, sugars, and pH within cells by optical techniques. Talley, *et al.*,[13] have extended this approach by inserting functionalized gold particles within cells that showed changes in the Raman spectrum with local pH changes. Again, these particles could be placed within cells to measure the distribution in acidity within cells, Figure 7. In order to measure

Figure 7. *Use of particles placed within cells to monitor intracellular analyte concentrations by surface-enhanced Raman spectroscopy. In this example of intracellular monitoring, a compound that shows different Raman spectra in its two ionic forms, is used to monitor the pH distribution within cells.[13]* ***(a)*** *Structure of the probe particle and Raman spectra at different pH's.* ***(b)*** *The pH behavior of Raman spectra.* ***(c)*** *Distribution of nanoparticles within cells.*

local enzyme activity within cells, Weissleder, *et al.*,[14] incorporated a probe polymer with an enzyme hydrolysable link between two fluorophores (see Figure 8).

With the increased amount of information afforded by these imaging techniques, software to manage and display this data in a meaningful fashion has become important. Several groups are developing appropriate software for this purpose.[15-18]

Government agencies are targeting technologies for improvement in intracellular imaging sensitivity. For example, the NIH recently funded nine centers to develop cellular imaging techniques. Descriptions of these research efforts as reported on the NIH Web site (<http://www.nigms.nih.gov/cellularimaging/index.html>) are quoted below.

OVERVIEW

The Exploratory Centers for the Development of High Resolution Probes for Cellular Imaging support multi-investigator teams to develop new technologies that enable higher-sensitivity biological imaging in living cells. Each of the nine centers will focus on different strategies for probe development, cellular delivery, probe targeting, and signal detection to improve detection schemes by a factor of 10 to 100. A major emphasis of this initiative is to apply novel, high-risk approaches to create fundamentally new probes with enhanced spectral characteristics. The ultimate goal is to develop probes and imaging systems that can be used to routinely achieve single-molecule sensitivity for imaging dynamic processes in living cells.

The centers are funded in conjunction with the NIH Roadmap for Medical Research as part of the "New Pathways to Discovery," an effort to advance our knowledge of biological systems by building a better toolbox for medical research. This initiative originated in NIGMS and was later adopted by the Roadmap. NIGMS currently supports seven of the centers as Roadmap-affiliated grants. Funding for all nine centers is expected to total approximately $25 million over four years ($6.8 million the first year).

1. ***Fluorescent Probes for Multiplexed Intracellular Imaging.*** *Kevin Burgess, Ph.D., Principal Investigator, Texas A&M University*

   Researchers from Texas A&M University and the University of Pennsylvania plan to create novel probe sets composed of multiplexed "through-bond energy transfer cassettes," using multiple, linked, donor-acceptor dye pairs that are optimized for cellular imaging. These probes, which efficiently absorb light at one wavelength, emit amplified fluorescent signals at different, resolvable wave lengths close to the red-infrared region, far removed from cellular autofluorescence. The dye cassettes will be specifically adapted for tracking interactions of proteins in cells, ultimately with single-molecule detection.

2. ***Sub-nm Dendrimer-Metal Nanoclusters as Ultrabright, Modular Targeted in vivo Single Molecule Raman and Fluorescence Labels*** • *Robert M. Dickson, Ph.D., Principal Investigator, Georgia Institute of Technology*

   Metal nanoclusters, composed of silver and gold atoms stabilized on organic dendrimers, exhibit strong, size-dependent emission throughout the visible and near-infrared spectrum. The spectral characteristics of these clusters—their small size (< 1 nm) and short and highly radiative

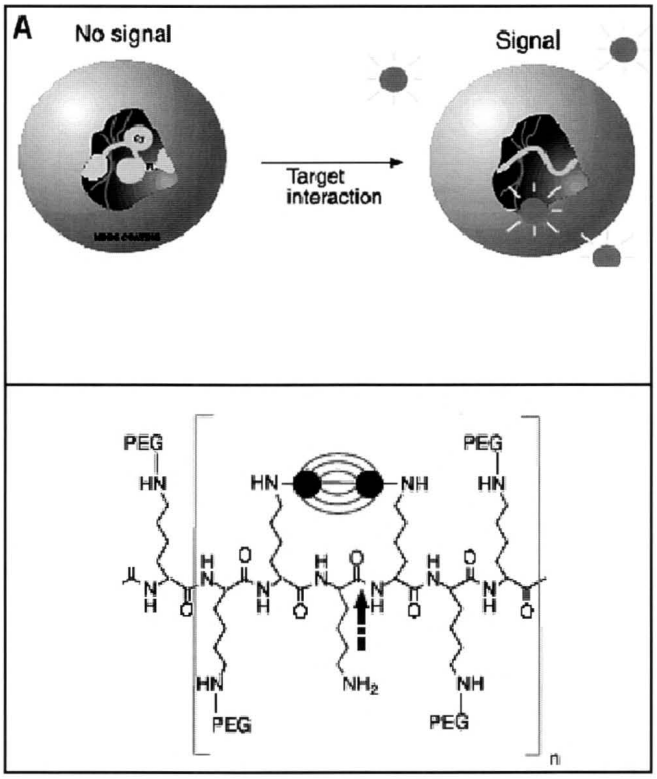


Figure 8. *Example of a polymer probe to determine enzyme activity within a cell. In this case the purpose was to monitor the activity of a proteolytic enzyme within cells. A special polymer substrate was created that contained the peptide bond that the enzyme cleaves and fluorophores (indicated by the circles) that self-quench when in close proximity. When the peptide bond is cleaved by the enzyme of interest, the fluorophores are separated and quenching is prevented. Thus, monitoring the appearance of fluorescence gives a measure of local enzyme activity. Upper panel: Schematic of the concept. Lower Panel: Example structure of the probe polymer to measure enzyme activity.*[14]

lifetimes—create signals that have the potential to be several orders of magnitude higher than conventional labels. Grantees from the Georgia Institute of Technology and Emory University plan to functionalize the nanoclusters for attachment to different biological targets and to develop single molecule imaging methods to facilitate detection of the signal inside cells.

3. ***Single-Molecule Fluorophores for Cellular Imaging*** • *William E. Moerner, Ph.D., Principal Investigator, Stanford University*

   A group from Stanford and Kent State University plans to synthesize and characterize a new class of highly emissive (dicyanodihydrofuran) fluorophores that exhibit large increases in signal when bound to rigid surfaces. The strategy for incorporating the probes into cells will be based upon the genetically encoded tetracysteine-biarsenical targeting system and then tested for single molecule specificity and detection in bacteria.

4. ***Bioaffinity Nanoparticle Probes for Molecular/Cellular Imaging*** • *Shuming Nie, Ph.D., Principal Investigator, Emory University and Georgia Tech*

   A collaborative group will develop a new class of polymer-encapsulated bioconjugated luminescent nanoparticles with enhanced optical properties, cellular delivery, and targeting/binding functions for real-time and multicolor imaging in living cells. The focus will be on core-shell semiconductor quantum dots because of their improved brightness, resistance against photobleaching, and simultaneous multicolor excitation. The researchers will test the probes and their ability to detect them in studies aimed at finding the subcellular locations of p53, nuclear factor B, and androgen receptor in living cells.

5. ***Probes for Quantitative Optical and Electron Microscopy*** • *David W. Piston, Ph.D., Principal Investigator, Vanderbilt University Medical Center*

   A group from Vanderbilt will develop new fluorescent probes in the visible and infrared spectral regions based on three approaches: genetically encoded proteins, lanthanide chelates, and nanocrystals (quantum dots). Each approach will be tested for imaging of a protein in the plasma membrane as well as an intracellular target. Subcellular resolution fluorescence imaging by widefield, deconvolution, confocal, and multi-photon excitation microscopy will be used to implement and test the new detection schemes based on spectral and time-gated resolution. To reach the highest resolution, the researchers will determine the utility and limitations of using the new probes for direct detection by electron microscopy for correlative imaging.

6. ***Imaging Single Proteins in vivo with Quantum Dots*** • *Sanford Simon, Ph.D., Principal Investigator, Rockefeller University*

   Researchers from the Rockefeller University plan to extend and optimize an in vivo trans-splicing and expressed-protein ligation approach to ligate quantum dot derivatives to cytosolic or integral membrane proteins. Their strategy includes development of a conditional protein trans-splicing approach that will allow probes to be ligated to the target following a designated functional interaction. The cellular fate of "activated" proteins will thus be monitored by a change in the signal emitted by the probe. The team intends to use these tools to study exocytosis and transport through nuclear pores.

7. ***Light-Activated Gene Expression in Single Cells*** • *Robert H. Singer, Ph.D., Principal Investigator, Albert Einstein College of Medicine*

   Investigators from the Albert Einstein College of Medicine will develop a photoactivatable gene that, upon exposure to light, begins transcription of visible nascent chains of RNA. The ecdysone response element and a caged, photoactivatable ecdysone gene into which an RNA reporter has been inserted, will be used. Gene expression will be initiated by uncaging the ecdysone in vivo by conventional and two-photon microscopy. The system will be engineered into cancer cells and then imaged intravitally in tumors. The dynamics of single RNA molecule movements and distribution will be monitored.

8. ***Library-Based Development of New Optical Imaging Probes*** • *Alice Ting, Ph.D., Principal Investigator, Massachusetts Institute of Technology*

   The investigators plan three parallel approaches to generate small-molecule and genetically encoded probes that can be targeted to specific RNA or protein sequences inside living cells. In the first, libraries of fluorophores will by synthesized in a combinatorial fashion and then screened for their ability to label small peptide motifs or RNA aptamers with high specificity. In the second approach, the natural bacterial enzyme biotin transferase will be re-engineered to catalyze covalent labeling of fluorescent probes to peptides inside cells. Third, a systematic approach using a

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